THE UNIVERSITY OF ALBERTA

IN VITRO STUDIES ON THE EFFECTS OF VALERIC ACID ON THE GROWTH AND METABOLISM OF RUMEN MICROORGANISMS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANIMAL SCIENCE

by

MING-FANG LI

EDMONTON, ALBERTA

MARCH, 1963

ABSTRACT

Experiments were conducted to study the effect of n-valeric acid on the growth and metabolism of washed cells of rumen microorganisms as well as of a pure culture of Bacteroides succinogenes S85, a cellulolytic ruminal bacterium.

Experiment I showed that valeric acid significantly increased the per cent cellulose digested and the weight of trichloroacetic acid insoluble nitrogen (TCA-N) formed by washed cells of rumen microorganisms during in vitro fermentation. There were no significant differences in levels of TCA-N or of cellulose digestion within replicates; however a highly significant difference was found between replicates for both factors. The percentage response obtained in per cent cellulose digested and TCA-N formed in the presence of valeric acid was not of the same magnitude, the per cent cellulose digested increased to a greater extent than TCA-N formed.

In Experiment II, the washed cells of rumen microorganisms were incubated with Na-valerate-1-C^{14}. Forty per cent of the total recovered activity appeared in the $Ba(OH)_2$ trap, the activity incorporated into the cellular fractions was about 4 per cent of the activity in the whole culture, and the major portion of the remaining activity in the supernatant was apparently in the form of radioactive carbon dioxide or other metabolites rather than as intact valerate-1-C^{14}. A high level of non-labelled valeric acid added to the supernatant did not affect the incorporation of the radioactive carbon into the cellular fractions.

Experiment III indicated that Bacteroides succinogenes S85 requires both n-valeric acid and isovaleric acid for growth. The peak growth of the organism was not increased by the presence of casein hydrolysate, but casein hydrolysate did promote early initiation of growth of the organism.

Acetic and propionic acids were the main volatile fatty acids formed by B. succinogenes S85 grown in a synthetic medium containing glucose as a source of energy. No butyric acid was formed. The results did not indicate the degree of utilization of n-valeric and isovaleric acids.

In Experiment IV, B. succinogenes S85 was cultured with Na-valerate-1-C^{14} in a synthetic medium. Only 1 per cent or less of the activity in the whole culture appeared in the $Ba(OH)_2$ trap. An appreciable amount of the radioactivity (approximately 1/10 to 1/3 of that in the whole culture) was incorporated into the bacterial cells, mainly in the lipid and protein fractions. In the protein fraction, the activity appeared mainly in lysine, arginine, aspartic acid, glutamic acid, proline and leucine; arginine, proline and glutamic acid had the highest relative specific activities. Possible metabolic pathways of utilization of valerate-1-C^{14} for synthesis of these active amino acids by B. succinogenes are discussed.

ACKNOWLEDGEMENTS

The writer wishes to thank Dr. L. W. McElroy, Head of the Department of Animal Science, for placing the facilities of the Department at his disposal and for invaluable assistance and guidance during the investigation and during the preparation of this manuscript. The assistance of Dr. A. R. Robblee, Professor of Poultry Nutrition, and Dr. J. M. Asplund, Assistant Professor of Animal Nutrition, is gratefully acknowledged.

The writer is also indebted to Dr. R. T. Berg, Associate Professor of Animal Genetics, for assistance with the statistical treatment of the data, to Dr. M. P. Bryant, Animal Husbandry Research Division, U.S.D.A., Beltsville, Maryland, for supply of the pure culture of Bacteroides succinogenes S85, and to Dr. W. G. Corns, Head of the Department of Plant Science, for permission to use the 'Micromil' window gas-flow counter. Mr. M. R. Batory assisted in the quantitative determination of amino acids in hydrolysates of bacterial protein.

This project was supported in part by a grant from the National Research Council of Canada.

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IN VITRO STUDIES ON THE EFFECTS OF VALERIC ACID ON THE GROWTH AND METABOLISM OF RUMEN MICROORGANISMS

INTRODUCTION

The ruminant is one of the best examples of symbiotic relationship between mammals and microorganisms. The rumen provides a suitable environment for continuous culture of the microbial population, and in return rumen microorganisms produce hydrolytic enzymes to degrade the insoluble feedstuffs such as cellulose into usable nutrients such as volatile fatty acids, and they also metabolize inorganic nitrogen into nutritious microbial protein and synthesize most of water soluble vitamins. Consequently ruminants can utilize roughage as the major energy source and they have no dietary requirement for essential amino acids and water soluble vitamins. It is apparent that the nutrient requirements of ruminants for efficient utilization of feeds are intimately associated with reactions occurring in the rumen as well as the essential and stimulatory nutrients of ruminal organisms.

In recent years, animal nutritionists and agricultural bacteriologists have studied the nutrient requirements as well as the ecology of rumen microorganisms. Some growth factors for these organisms have been identified. The increase of the cellulose digested, coupled with the increase of the cellular protein formed in artificial rumen flasks, indicates that valeric acid is an important growth factor for rumen microorganisms. The requirement of this short chain fatty acid for growth of ruminal cellulolytic bacteria has been well studied. However, from the viewpoint of scientific investigation of a nutrient it is evident that to study the growth is not enough, of equal importance is to study the metabolic function of the nutrient, whether it is rapidly metabolized as energy source or if it is utilized as a precursor

for the synthesis of cellular constituents. Experiments were undertaken to study the effect of valeric acid on growth of the washed cells of rumen microorganisms and on a purified culture of Bacteroides succinogenes S85, a ruminal cellulolytic bacterium, as well as to study the distribution of C^{14} from valerate-1-C^{14} in the various cellular fractions such as lipid, nucleic acid, and protein, of the washed cells and B. succinogenes S85.

REVIEW OF LITERATURE

Formation of valeric acid by rumen microorganisms

The presence of valeric acid in the rumen has been reported; the level present may be affected by the ration fed and by time after feeding. Gray et al. (1951) indicated that valeric acid constituted about 2.6% of total short chain fatty acid produced in the rumen of sheep on a ration of wheaten hay. Annison (1954) and Stewart et al. (1958) showed that valeric acid levels in rumen fluid vary as the result of dietary changes; readily digestible carbohydrate and protein seem to favor valeric acid formation. Stewart et al. (1958) noted that time after feeding influences the levels of valeric acid in rumen contents; the proportion present increases slightly immediately after feeding.

In studies with a ruminal coccus Elsden et al. (1951) found that addition of acetate to a pyruvate medium doubled the yields of n-butyrate, n-valerate and n-caproate while addition of propionate gave a ten-fold increase in n-valerate. There was at the same time a disappearance of the added acetate or propionate. These results were taken as evidence that a C_2 fragment of pyruvate combined with the added fatty acid and the resulting compound was then reduced by a hydrogenase system. This postulation regarding the method of synthesis of higher fatty acids in the rumen was confirmed by the tracer studies of Gray et al. (1952) who incubated labelled fatty acids with rumen ingesta. When acetic acid labelled with C^{14} in the carboxyl group was added to rumen ingesta prior to incubation in vitro, radioactive carbon appeared in the C_4 to C_6 volatile fatty acids. When labelled propionic acid was used, radioactive carbon appeared in valeric but not in butyric acid.

The postulation that valeric acid may be synthesized by rumen organisms by condensation of C_2 and C_3 fatty acids was supported by the work of Ladd (1957) who found that in the fermentation of DL-2C^{14}-lactate by a

rumen organism the CO_2 produced was not labelled and that the acetate and propionate formed were labelled in the carboxy and methylene positions respectively. Based on his results, Ladd suggested the following equations to explain the formation of valeric acid from lactate.

$$CH_3 \cdot {}^{14}CHOH \cdot CO_2H + 2(H) \longrightarrow CH_3 \cdot {}^{14}CH_2 \cdot CO_2H$$

$$CH_3 \cdot {}^{14}CHOH \cdot CO_2H \longrightarrow CH_3 \cdot {}^{14}CO_2H + CO_2 + 4(H)$$

$$2CH_3 \cdot {}^{14}CO_2H + 4(H) \longrightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot {}^{14}CO_2H$$

$$CH_3 \cdot {}^{14}CH_2 \cdot CO_2H + CH_3 \cdot {}^{14}CO_2H + 4(H) \longrightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot CH_2 \cdot {}^{14}CO_2H$$

$$3CH_3 \cdot {}^{14}CO_2H + 8(H) \longrightarrow CH_3 \cdot {}^{14}CH_2 \cdot CH_2 \cdot {}^{14}CH_2 \cdot CH_2 \cdot {}^{14}CO_2H$$

Further evidence in support of this mechanism of valerate formation was supplied by Van Campen and Matrone (1960) who found that the radioactive carbon of C^{14}-labelled bicarbonate was mainly incorporated into propionate and that the specific activity of valerate always exceeded that of either acetate or butyrate.

However, accumulated evidence indicates that carbohydrates are not the only source of valeric acid in the rumen; protein or amino acids may also be involved. el-Shazly (1952a) reported that the main reaction products obtained from the incubation of rumen bacteria with casein hydrolysate were NH_3, CO_2, and volatile fatty acids. The increase in NH_3 concentration was positively correlated with the formation of isobutyric and C_5 acids. Analysis of the fermentation mixture by paper chromatography indicated a decrease in the concentration of all amino acids, and the consistent appearance of a new compound which was subsequently identified as δ-amino valeric acid. Further investigation led el-Shazly (1952b) to conclude that δ-amino valeric acid is formed from proline via the Stickland reaction. el-Shazly postulated that valine, leucine, and isoleucine could act as hydrogen donors, being oxidized to branched chain C_4 and C_5 acids; proline could serve as the hydrogen acceptor, being reduced to δ-amino valeric acid. Thus n-valeric acid could arise from arginine, ornithine, proline or lysine via δ-amino

valeric acid. This suggestion was confirmed by the tracer studies of Dehority et al. (1958). Labelled δ-amino valeric acid was isolated from in vitro fermentation mixtures to which L-proline-C^{14} had been added. The part of the C^{14} activity recovered as fatty acid at the end of a 24-hour incubation was found in the C_5 fraction.

Valeric acid as a growth factor for rumen microorganisms

The presence in rumen juice of an unidentified nutritional factor(s) for rumen microorganisms has been described by several investigators (Hungate, 1950; Burroughs et al., 1950b; Huhtanen et al., 1952; Doetsch et al., 1952; Bentley et al., 1953, 1954a; Garner et al., 1954; and McNeill et al., 1954).

Bentley et al. (1954b, 1954c) reported that a volatile fraction from acidified rumen juice, but not from alkaline rumen juice, had nearly the same in vitro cellulolytic stimulatory effect as centrifuged rumen juice. The behavior of the active material during distillation suggested that this substance could be a steam volatile fatty acid(s). This postulation was confirmed by the fact that the addition of short chain volatile fatty acids to the fermentation medium increased cellulose digestion. Caproic and n-valeric acid were the most active although isovaleric and isobutyric acid were found to have some stimulatory effect. Acetic, propionic and butyric acid were inactive as well as the C_7 - C_{10} straight chain fatty acids tested. Although caproic acid was present in trace amounts, the in vitro cellulolytic stimulatory activity of the volatile fatty acid fraction of rumen juice was mainly due to valeric acid (Bentley et al., 1955). From the fact that, besides increasing cellulose digestion, valeric acid increased the amounts of TCA-insoluble nitrogen formed in fermentation flasks, the authors suggested that valeric acid increased the numbers of bacteria.

The cellulolytic stimulatory effects *in vitro* of yeast, casein hydrolysate and alfalfa meal have been studied by several workers. Dehority *et al*. (1957) demonstrated that at least a part of the stimulation is attributable to amino acids such as valine, proline and leucine, which can be converted to branched and straight chain fatty acids in the rumen.

Several workers, including Burroughs *et al*. (1950a) have shown that cellulose digestion is depressed in the presence of excessive amounts of readily fermentable carbohydrate. Under these same conditions Cline *et al*. (1958) showed that increased levels of valeric acid are formed. It has been postulated that amylolytic organisms either do not require valeric acid for growth or that their requirement is much lower than that of cellulolytic organisms. Cline and co-workers concluded that the disappearance of valeric acid added to fermentation tubes coupled with increased cellulose digestion and synthesis of bacterial protein implies that valeric acid is an important metabolite for cellulolytic rumen microorganisms.

Isolation and culture of strains of anaerobic cellulolytic bacteria was started about 30 years ago by Cowles and Rettger (1931) who laid the foundations for procedures employed at the present time. Hungate (1947) first isolated, maintained, and characterized cellulolytic bacteria morphologically and physiologically. A number of ruminal cellulolytic bacteria have since been isolated and studied by several workers (Hungate, 1950; Sijpesteijn, 1951; Bryant and Burkey, 1953; Bryant and Doetsch, 1954). Based on the numbers present in rumen juice and the rate of cellulose digestion *Bacteroides succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* seem to be among the most important cellulolytic bacteria in the rumen (Hungate, 1950; Bryant and Doetsch, 1954; Bryant *et al*., 1959; Bryant and Robinson, 1961a).

It has been shown that isovaleric, but not n-valeric acid is essential for the growth of ruminal cellulolytic cocci (Allison and Bryant, 1958; Bryant and Robinson, 1961a) whereas *B. succinogenes* requires both branched

and straight chain fatty acids. Bryant and Doetsch (1955) indicated that the growth factor for B. succinogenes present in the volatile fatty acid fraction of rumen fluid consisted of two components, one a branched chain fatty acid, either isobutyric, isovaleric or 2-methyl butyric, and the other a straight chain fatty acid. Any of the C_5 to C_8 series of fatty acids could be used as the second component. This two-component volatile fatty acid requirement of B. succinogenes was further demonstrated by Bryant et al. (1959).

Because ruminal cellulolytic cocci do not require n-valerate for growth it seems logical to postulate that the cellulolytic stimulatory effect of valeric acid in fermentations involving mixtures of rumen organisms is mainly due to its effect on the growth of B. succinogenes. Bryant et al. (1959) have also demonstrated that this organism has a relatively great capacity to synthesize cellular constituents when grown in media in which ammonia and glucose or cellulose are the main sources of nitrogen and energy respectively.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

Experiments were conducted *in vitro* to study the effect of valeric acid on the growth and cellulolytic activity of rumen microorganisms and to determine the distribution of C^{14} from labelled sodium valerate-1-C^{14} in the organisms. The specific areas studied are listed below.

Experiment I. Effects of valeric acid on growth of ruminal microorganisms and cellulose digestion in the artificial rumen.

Experiment II. Distribution of labelled carbon from sodium valerate-1-C^{14} in the cellular fractions of washed cells of rumen microorganisms.

Experiment III. Effect of valeric acid on growth of *B. succinogenes* S85.

Experiment IV. Distribution of labelled carbon from sodium valerate-1-C^{14} in the cellular fractions of *B. succinogenes* S85.

Experiment I
Effects of Valeric Acid on Growth of Ruminal Microorganisms and Cellulose Digestion in the Artificial Rumen

Introduction and object

The stimulatory effect of valeric acid on cellulose digestion *in vitro* with an associated increase in formation of trichloroacetic acid (TCA) insoluble nitrogen has been observed (Bentley *et al.*, 1955; Cline *et al.*, 1958). However, no well replicated experiments appear to have been done to determine whether a relationship exists between the amount of TCA-insoluble nitrogen formed and the per cent of cellulose digested by washed ruminal cells grown in media with and without added valeric acid. Experiment I was conducted to investigate the effects of valeric acid on the growth of microorganisms and on the digestion of cellulose and to secure data on the repeatability of results obtainable with washed rumen cell inocula. Four replicates

were completed, each with inoculum prepared from samples of rumen ingesta taken from one cow at weekly intervals.

Methods

Preparation of inocula

Rumen ingesta from a rumen fistulated cow, fed a ration of high quality alfalfa hay, served as the source of inocula. Preparation of the washed-cell inocula was patterned after the method described by Johnson et al. (1958). Ten pounds of rumen ingesta were taken six hours after feeding and the liquid therein was removed immediately by pressing and was discarded. The remaining pulp was mixed with 2 liters of phosphate buffer (1.059 g Na_2HPO_4, 0.436 g KH_2PO_4, and 0.01 g cysteine-HCl per liter) which had been warmed to a temperature of 39°C and saturated with carbon dioxide. The mixture was stirred gently and the liquid was removed by pressing.

The resultant phosphate buffer extract was transported from the barn to the laboratory in a thermo-container, and was centrifuged in a Sharples super-centrifuge at approximately 30,000 r.p.m. The sediment which collected above a line 2 inches from the bottom of the celluloid liner of the centrifuge was suspended in 1700 ml of the phosphate buffer and was centrifuged again. The cells collected on the liner were resuspended in 300 ml of buffer. This suspension was used as inoculum for the in vitro fermentations.

Fermentation procedure

Fermentations were carried out in round bottom centrifuge tubes of 90 ml capacity. Each tube contained the quantity of basal medium listed in Table 1 plus 0.2 g of cellulose* and distilled water to give a total volume of 45 milliliters. The centrifuge tubes were fitted with gas inlet and outlet tubes of 4 mm diameter and carbon dioxide was allowed to bubble through the contents of the tubes for 30 minutes. The tubes were then inoculated with 5.0 ml of inoculum, placed in a water bath and incubated for 56 hours at 39°C.

*Solka Floc SW-40-A, Brown Forest Products Ltd., Montreal, P.Q.

Table 1
Basal medium for *in vitro* fermentation

Component	Volume per tube ml
Na_2CO_3 (200 mg/ml)	0.5
10% glucose solution	0.5
Biotin (10 µg/ml)	1.0
p-aminobenzoic acid (100 µg/ml)	0.25
Urea (126 mg/ml)	0.5
$FeCl_3.6H_2O$ (4.4 mg/ml)	0.5
$CaCl_2$ (5.29 mg/ml)	0.5
Mineral mixture*	10.0

*Ten liters of the mineral mixture (pH 6.55) contained the following in grams: NaH_2PO_4, 56.5; Na_2HPO_4, 54.5; KCl, 21.5; NaCl, 21.5; $MgSO_4.7H_2O$, 5.82 and Na_2SO_4, 7.5.

Determination of TCA-insoluble nitrogen

A method similar to that used by Cline *et al.* (1958) was followed in the determination of TCA-insoluble nitrogen (TCA-N). The contents of the tubes after fermentation were precipitated by adding 12 ml of 50% trichloroacetic acid to each tube. The mixture was allowed to stand 18 to 24 hours at 5°C. The precipitate was separated by centrifuging for two hours at about 3000 r.p.m. in a refrigerated centrifuge*. The supernatant was decanted, and the precipitate washed three times with 10% trichloroacetic acid after centrifuging as before. The washed precipitate from each tube was transferred to a micro-Kjeldahl flask for nitrogen analysis (A.O.A.C., 1960).

Measurement of cellulose digestion

The method of Crampton and Maynard (1938), with minor modifications, was used to determine per cent cellulose digested. The fermented tubes were centrifuged at 2200 r.p.m. for 8 minutes in a refrigerated centrifuge, the

*MSE Model P.

supernatant was discarded and 20 ml of 80% acetic acid and 2 ml of concentrated nitric acid were added to the residue. Digestion was conducted by immersing the tubes in a boiling water bath for a period of 30 minutes (Donefer et al., 1960).

At the end of the digestion period, the tubes were removed from the water bath and allowed to cool for 5 minutes. Twenty-five ml of 95% ethanol were added to each tube after which the contents were quantitatively transferred to and filtered through Selas crucibles of extremely coarse porosity. After repeated washing with 95% ethanol, the cellulose left in the crucible was washed with approximately 5 ml of acetone. The crucibles and contents were then dried at 110°C for 30 minutes and weighed. The cellulose remaining was ashed by placing the crucibles and contents in a muffle furnace (550-600°C) for two hours. After cooling, the crucibles were weighed. Cellulose was estimated to be equal to the loss of weight on ashing.

Experimental design

A three by four factorial design was used in the experiment with three treatments, four replicates and four observations in each subplot. The treatments used consisted of three levels of valeric acid; 0, 7.5 and 15 mg per 100 ml of medium. In each replicate, 24 fermentation tubes were arranged in four rows of six tubes each as shown in Figure 1.

1	2	3	1	2	3
2	3	1	2	3	1
3	1	2	3	1	2
1	2	3	1	2	3

TCA-insoluble nitrogen | Cellulose

Figure 1. Arrangement of tubes during fermentation. Tubes numbered 1, 2 and 3 contained 0, 7.5 and 15 mg of valeric acid per 100 ml of medium respectively.

At the end of the incubation period 12 tubes were used for TCA-N determinations and 12 for cellulose digestion as indicated in Figure 1. Four extra tubes, with the same amount of medium and inoculum, stored at 5°C, were used as controls.

Results and Discussion

The addition of valeric acid to the medium resulted in an increase ($P < .01$) in the level of TCA-N produced during incubation and in the percentage of cellulose digested (Table 2). In each replicate, the responses to valeric acid were approximately the same with the addition of either 7.5 or 15 mg of valeric acid per 100 ml of medium.

Table 2
Cellulose digested and bacterial synthesis by rumen microorganisms *in vitro* using different levels of valeric acid added to the basal medium

Valeric acid mg per 100 ml	TCA-N (mg)*			Cellulose digestion (%)*			% Cell. dig. : mg TCA-N		
	0	7.5	15	0	7.5	15	0	7.5	15
Replicate 1	2.53	3.55	3.52	36.9	70.0	75.1	15:1	20:1	21:1
Replicate 2	2.31	4.28	3.80	26.1	84.0	89.3	11:1	20:1	24:1
Replicate 3	2.51	3.68	3.62	47.2	75.5	70.9	19:1	20:1	20:1
Replicate 4	3.25	4.38	4.22	64.3	86.2	77.2	20:1	20:1	18:1
Average	2.65	3.97	3.79	43.6	78.9	78.1	16:1	20:1	21:1

*Mean of four observations.

Since the addition of 7.5 mg of valeric acid per 100 ml gave as high levels of TCA-N and of cellulose digestion as 15 mg, it would appear that the optimum level may be close to 7.5 milligrams. This is lower than the optimum level reported by Bentley *et al.* (1955), who found that a maximum response was elicited by the addition of 10 mg of valeric acid per 100 ml of medium, with levels of 20 or 50 mg depressing cellulose digestion.

Higher percentages of cellulose digestion and lower levels of TCA-N were observed in this experiment than were reported by Bentley *et al.*, but the experiments are not strictly comparable since less than half of the level of cellulose employed by Bentley and co-workers was used in the present experiment.

While the addition of valeric acid caused a response in levels of both TCA-N and cellulose digestion the percentage response obtained in each was not of the same magnitude (Table 2). The addition of 7.5 mg of valeric acid per 100 ml of medium increased TCA-N by a factor of 1.5 (2.65 to 3.97 mg) and increased cellulose digestion by a factor of 1.8 (43.6 to 78.9%) as compared to results obtained with the unsupplemented medium. The fact that cellulose digestion increased to a greater extent than TCA-N suggests that the increase in microbial population, as measured by TCA-N, was probably due mainly to increased numbers of cellulolytic bacteria, such as *B. succinogenes*, which require valeric acid as a growth factor, and that other bacteria present may not have increased to any extent. This would agree with the postulation of Cline *et al.* (1958) that either ruminal amylolytic bacteria do not require valeric acid, or their requirement is much lower than that of cellulolytic bacteria.

The results of statistical analysis of the data indicate that there were no significant differences in levels of TCA-N or of cellulose digestion within replicates; however, a highly significant ($P < .01$) difference was found between replicates for both factors (Table 2). The differences noted may have been due to differences in the inocula used in the four replicates or may have resulted from other variables in the fermentation environment. Although the rumen ingesta were collected and the inocula were prepared under essentially the same conditions, the final inocula obtained may have varied considerably from week to week.

Summary

1. The addition of valeric acid to a purified medium resulted in an increase in levels of TCA-N produced and of cellulose digested during *in vitro* fermentations. In this experiment the optimum level of valeric acid appeared to be 7.5 mg per 100 ml of medium.

2. The addition of valeric acid to the fermentation medium resulted in a greater percentage increase in cellulose digested than in weight of TCA-N produced.

3. Although the procedure used gave consistent results within replicates a highly significant variability occurred between replicates.

Experiment II
Distribution of Labelled Carbon from Sodium Valerate-1-C^{14} in the Cellular Fractions of Washed Cells of Rumen Microorganisms

In agreement with the results of others, it was observed in Experiment I that the inclusion of valeric acid in the medium caused an increase in cellulose digestion coupled with an increase in TCA-N during fermentation. These results demonstrate that valeric acid is a growth factor for rumen microorganisms but do not indicate whether it is utilized simply as a rapidly metabolized energy source or as a carbon source for synthesizing specific cellular constituents. The present and succeeding experiments were planned to study the possible role of valeric acid in the metabolism of rumen microorganisms using sodium valerate-1-C^{14} as a tracer.

Materials and Methods

Sodium valerate-1-C^{14} (specific activity: 0.86 mc/mmole) was purchased from a commercial source*.

Inoculum

The rumen ingesta was obtained from the same fistulated cow fed the same ration of alfalfa hay as in Experiment I. The inoculum was prepared by the same procedures as those described in Experiment I, but the cells were resuspended only once instead of twice.

In vitro fermentation

The basal medium and substrate were the same as those used in Experiment I. The in vitro system was similar to that of Experiment I, except that fermentation was conducted in 250 ml Erlenmeyer flasks connected in series. After the experimental media were inoculated the CO_2 inlet tube was closed, and the outlet tube was connected to a series of flasks containing a saturated solution of $Ba(OH)_2$. The fermentation was carried out at 39°C for 24 hours.

*New England Nuclear Corporation, Boston, Mass.

Fractionation of cellular components

At the end of the fermentation period the cells were harvested by centrifugation, and washed three times with physiological saline, each time using an amount equivalent to approximately one-third of the original volume of whole culture. The washed bacterial solids were chemically fractionated into lipid, nucleic acid, and protein portions according to the method of Abelson et al. (1952). The lipid fraction was obtained by extraction of the bacterial solids with a quantity of hot 80% ethanol equal to about one-quarter of the volume of whole culture followed by two extractions with similar volumes of ether-ethanol (1:3) mixture. After each extraction the mixture was centrifuged, the supernatant was decanted and the lipid extracts thus obtained were combined for estimation of radioactivity.

The bacterial solids were further treated with 5% trichloroacetic acid (TCA) solution, equal to about one-sixth of the volume of the whole culture, at 90°C for 15 minutes, and washed once with approximately the same amount of cold 5% TCA solution. The TCA extracts were considered to be the nucleic acid fraction.

The residual solids (protein) were hydrolyzed in 10 ml of 6N HCl on a steam bath for 24 hours. To remove the HCl the protein hydrolysates were evaporated to about 1 ml on a steam bath; 10 ml of distilled water were added and evaporation to 1 ml repeated, after which they were diluted to 10 ml with 50% isopropyl alcohol. The nitrogen contents of 1 ml portions of the protein hydrolysates were determined in duplicate by the micro-Kjeldahl method (A.O.A.C., 1960).

Estimation of radioactivity

A 50 microliter sample of each fraction drawn with a micro-pipette was spread evenly on an aluminum planchet. The radioactivity of each of these samples was measured in a windowless gas flow counter*. Since the

*Nuclear Chicago Model C-1108

samples used for counting weighed less than 1 mg/cm^2 after drying, corrections were made only for background counts, not for self-absorption.

Chromatographic separation of amino acids

The protein hydrolysates in 50% isopropyl alcohol solution were stored at -18°C for at least 12 hours before subjection to one-dimensional ascending paper chromatography on Whatman No. 1 filter paper. A mixture of n-butanol-acetic acid-water (4:1:5) was used as the resolving solution. The paper was sprayed with 1% ninhydrin in n-butyl alcohol to detect the amino acids. Identification of the amino acids in the hydrolysates was accomplished by running known amino acids and the hydrolysate samples on the same filter paper sheets (Hughes et al., 1958).

Radioautographs

Radioautographs of the paper chromatograms were prepared employing Ilford 'no screen' X-ray film, and an exposure time of 4 weeks (Katz and Chaikoff, 1954). The paper chromatograms were stapled to the X-ray film and were stored between two pieces of sponge-lined plywood, in complete darkness, for a period of 4 weeks. The films were then developed in a tray of Kodak D19 developer for 5 minutes after which they were placed in an acetic acid bath for 30 seconds, in Kodak-acid fixing solution for 5 minutes and in running water for 30 minutes. The developed films were then dried.

Trial 1

Object

To trace the distribution of C^{14} in the cellular fractions obtained after *in vitro* fermentations of rumen microorganisms in a medium containing valerate-1-C^{14}.

Experimental

The fermentation system was arranged as shown in Figure 2.

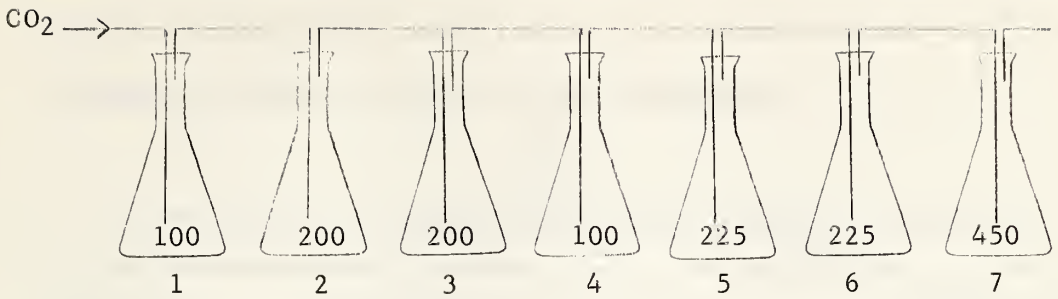

Figure 2. Arrangement of fermentation flasks. The numbers shown in the flasks indicate the volume in ml of fermentation mixture or $Ba(OH)_2$ solution.

The first four flasks, connected in series, contained the same basal medium, substrate and inoculum except that 6.5 mg of sodium valerate-1-C^{14} per 100 ml of medium was added to flask 2 and non-labelled valeric acid was added to the other flasks at a level of 7.5 mg per 100 ml of basal medium. Flasks 5-7 contained a saturated solution of $Ba(OH)_2$.

Radioactivities of the contents of each of flasks 1-4 and of the pooled contents of flasks 5-7 were determined by measuring the activity of a 50 microliter sample of each after a 24-hour fermentation period. The bacterial cells in the combined contents of flasks 1 and 2 were then harvested and separated into cellular fractions and radioactivity in the supernatant, wash, and cellular fractions was determined as described under 'Materials and Methods'.

Results and discussion

Following incubation, radioactivity was distributed throughout all of the flasks in the series (Table 3). A decrease of approximately 22 ml of the radioactive medium in flask 2 with a corresponding increase in volume of flask 1 was observed after fermentation. The radioactivity present in flask 1 was obviously caused by back flow of medium from flask 2, while that found in flasks 3-7 was due to the flow of radioactive CO_2 or other gases

formed in flasks 1 and 2 during fermentation. The high level of activity present in the $Ba(OH)_2$ trap indicates that the valerate-1-C^{14} was extensively oxidized to radioactive CO_2 during fermentation.

Table 3
Distribution of radioactivity after *in vitro* fermentation

Flask No.	Treatments	Radioactivity (cpm)
1	Non-labelled valeric acid	6,250,000
2	Valerate-1-C^{14}	46,784,000
3	Non-labelled valeric acid	639,000
4	Non-labelled valeric acid	239,000
5-7	$Ba(OH)_2$ trap	38,362,000

Because high radioactivity originating from back flow from flask 2 was found in flask 1, the contents of the two flasks were pooled before the distribution of labelled carbon in the whole culture, supernatant and cells was determined (Table 4).

Table 4
Distribution of radioactivity in pooled contents of flasks 1 and 2

Fractions	Distribution of C^{14}	
	Radioactivity (cpm)	Per cent
Whole culture	53,034,000	100
Supernatant and wash	46,688,000	88.0
Washed bacterial cells	2,236,000	4.2
Lipid	1,814,000	3.4
Nucleic acid	31,000	0.1
Protein	391,000	0.7
Total recovered activity		92.2

Incorporation of activity from the labelled valerate into the bacterial cells was low; approximately 96% of the recovered activity was found in the supernatant and 4% in the cellular fractions. The distribution of radioactivity in the cellular fractions indicated that approximately 80% of the activity was present in the lipid portion, with 1.4 and 18% in the nucleic acid and protein fractions respectively.

The distribution of radioactivity in the supernatant and in the lipid fraction from the washed bacterial cells gave little indication of the form in which the C^{14} was present. When an aliquot of the supernatant was acidified with HCl there was a loss of 85% of the radioactivity. This suggests that the activity present was probably in the form of carbonate which was evolved as $C^{14}O_2$ when the material was acidified. When the lipid fraction was distilled under acid conditions on a steam bath, 64% of the radioactivity was recovered in the non-volatile portion. Since valeric acid is volatile, it would appear that changes occurred during fermentation which resulted in incorporation of C^{14} into non-volatile lipids.

Low cellular incorporation of C^{14} plus evidence of extensive formation of radioactive CO_2 suggests that valerate-1-C^{14} was utilized principally as a readily available energy source rather than as a direct precursor of cellular constituents. Since CO_2 may be used in biosynthesis by some organisms it is not known whether the C^{14} in the cellular fractions was derived directly from valerate-1-C^{14} or arose from incorporation of $C^{14}O_2$ into cellular compounds.

One radioactive amino acid was revealed by the radioautograph made from the one-dimensional chromatogram. This will be discussed under Trial 2 (Figure 4) in relation to the corresponding radioautograph obtained in the second trial.

Summary

1. The incorporation of labelled valerate-1-C^{14} into the bacterial cells was low; most of that present was found in the lipid fraction.

2. The results suggest that valerate-1-C^{14} is utilized principally as a source of energy rather than as a precursor of cellular constituents.

Trial 2

Object

The results of the previous trial indicated that valerate-1-C^{14} was utilized by rumen organisms during fermentation. A small amount of C^{14} was incorporated into the cellular constituents but it appeared that most of the valerate-1-C^{14} was converted, during the fermentation, to $C^{14}O_2$ or other metabolites. Since it has been demonstrated that certain organisms may utilize CO_2 for biosynthesis (Abelson *et al.*, 1952; Huhtanen *et al.*, 1954), it was not possible to determine whether the cellular activity arose directly from valerate-1-C^{14} or from $C^{14}O_2$.

This trial was designed to determine whether radioactive metabolites which had been produced from valerate-1-C^{14} during a previous fermentation could be used for synthesis of cellular constituents or whether intact valerate-1-C^{14} was required for incorporation of radioactivity into the cellular components.

Experimental

The *in vitro* fermentation system was arranged as shown in Figure 3.

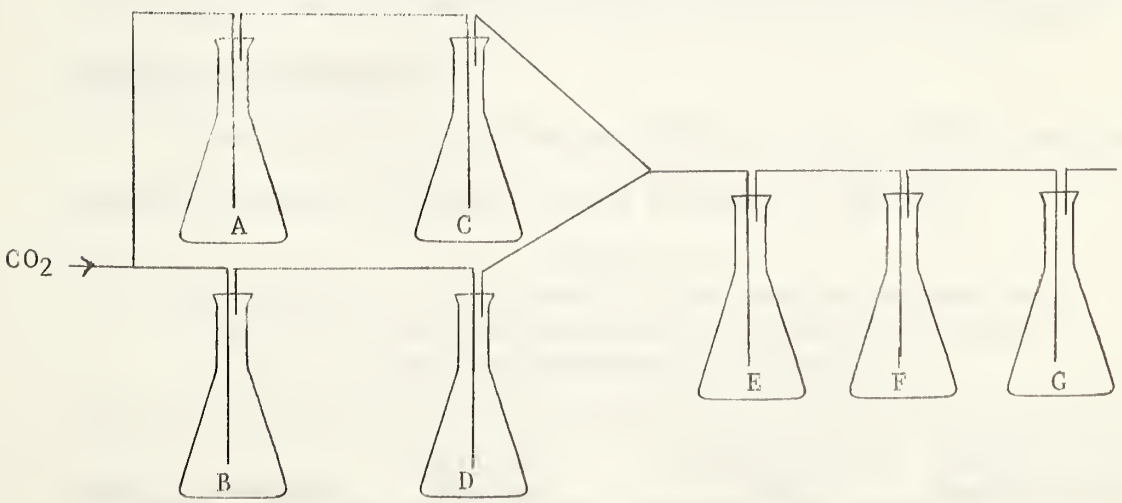

Figure 3. The arrangement of fermentation flasks in the study of dilution effect of non-labelled valeric acid on the cellular incorporation of the radioactive carbon in the supernatant of Trial 1 by rumen microorganisms.

Flasks A and B were used for the experimental treatments. Flask A contained a fermentation mixture consisting of 125 ml (17,072,510 cpm) of the supernatant from Trial 1, and 100 ml of freshly prepared basal medium. Flask B contained the same amount of supernatant and basal medium but non-labelled valeric acid was added at a level of 190 mg/100 ml of fermentation mixture. It was theorized that the addition of a high level of non-labelled valeric acid to the fermentation medium would reduce the direct incorporation of valerate-1-C^{14} into cellular components during fermentation. Flasks C and D, included in the system to maintain a slight positive CO_2 tension in flasks A and B, contained 100 ml of basal medium and 7.5 mg of non-labelled valeric acid. Flasks E, F and G contained saturated $Ba(OH)_2$ solution.

The flasks were inoculated with washed bacterial cells and were incubated at 39°C for 24 hours. At the end of the fermentation period the cells in flasks A and B were harvested and fractionated and the radioactivity of each fraction was estimated by the procedures used in the previous trial.

The cell protein hydrolysates from Trial 1 and the combined hydrolysates from Trial 2 were chromatographed on the same sheet and radioactivity incorporated into the amino acids was detected by radioautographs.

Results and discussion

The distribution of radioactivity in the cellular fractions derived from the contents of flasks A and B is shown in Table 5.

Table 5
The effect of a high level of non-labelled valeric acid on the cellular incorporation of radioactive carbon from the supernatant of Trial 1.

Flask	Treatment	Cellular fraction	Radioactivity cpm	Specific activity cpm/μg of nitrogen
A	Supernatant	Lipid	329,000	-
		Nucleic acid	46,000	-
		Protein	569,000	49
B	Supernatant + valeric acid	Lipid	295,000	-
		Nucleic acid	48,000	-
		Protein	529,000	52

It is apparent that the addition of a high level of non-labelled valeric acid to the fermentation medium had no effect on incorporation of C^{14} into the cellular components. The lack of any effect, either with respect to total activity of each of the fractions or to specific activity of the protein fraction, indicates that the activity noted did not arise directly from valerate-1-C^{14} in the medium. If it had, the dilution effect of added non-labelled valeric acid would have decreased C^{14} levels in one or more of the fractions obtained from flask B. The radioactivity present must therefore have been derived from $C^{14}O_2$ or other radioactive components present in the medium.

The distribution of activity in the cellular fractions differed from that of Trial 1. In Trial 1 the lipid fraction contained about 80% of the incorporated activity and the protein fraction contained less than 20 per cent. In contrast, in this trial the lipid fraction contained less than 35% of the radioactivity and the protein fractions contributed more than 60% of the activity. The pattern of C^{14} distribution noted in this trial is in agreement with the observations of Roberts *et al.* (1955) which showed that when *E. coli* was cultured with $NaHC^{14}O_3$ the majority of the cellular incorporated activity was recovered in the protein fraction.

The distribution of amino acids as indicated by paper chromatography and a radioautograph of the chromatograms from Trials 1 and 2 is shown in Figure 4.

No particular differences were noted in the paper chromatograms; however, radioautographs of the chromatograms showed marked differences. The radioautograph of the chromatogram of the protein hydrolysate from Trial 1 showed only one amino acid with radioactivity while that from Trial 2 showed at least nine amino acids with radioactivity. This indicates much more extensive incorporation of C^{14} into the protein. Since the supernatant of Trial 1 was used as the radioactive source for Trial 2, and

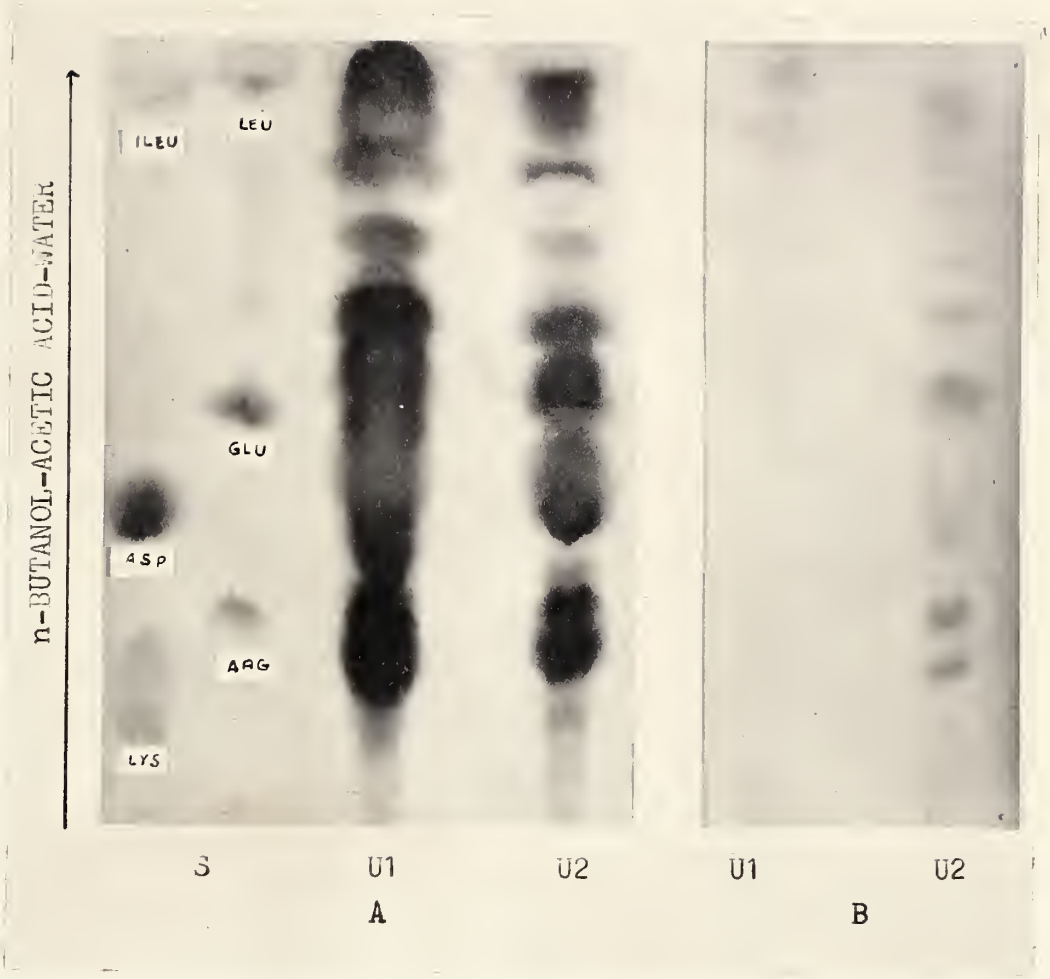

Figure 4. (A) Chromatograms. S, standard; U1, hydrolysate from Trial 1; U2, hydrolysate from Trial 2.
(B) Radioautographs of the chromatograms.

since the media and inocula differed in the two trials, it might be theorized that the differences in extent of incorporation of C^{14} into amino acids may have been caused by the growth of different types of organisms. It is possible that the predominant organism(s) in Trial 1 had limited ability to utilize either valerate-1-C^{14} or $C^{14}O_2$ for growth whereas the predominant organism(s) in Trial 2 apparently were able to do so.

Summary

1. Indirect evidence was obtained which indicated that C^{14} activity incorporated into cellular fractions during *in vitro* fermentation did not arise directly from valerate-1-C^{14} but rather from $C^{14}O_2$ or other radioactive components in the medium.

2. Radioautographs of chromatograms of cellular protein hydrolysates showed that C^{14} activity was present in at least nine amino acids in this trial but only in one in the previous trial.

Experiment III
Effect of Valeric Acid on Growth of Bacteroides succinogenes S85

Introduction

As is indicated by the results of Experiment II, it appears impossible to follow the pathways of valeric acid metabolism by employing valerate-1-C^{14} as a tracer in cultures as complex as are those of washed ruminal cells. A pure culture of one appropriate rumen bacterium would of course provide a less complex biological system in which to study the role of valeric acid in the metabolism of rumen microorganisms.

As was indicated under 'Review of Literature', B. succinogenes is not only an important ruminal cellulolytic agent, but one with a relatively high capacity to synthesize cellular constituents when grown in media containing CO_2 with cellulose or glucose as the energy source and NH_3 as the main source of nitrogen. Also, in contrast to the case of a number of other important ruminal cellulolytic organisms, n-valeric acid is essential for growth of B. succinogenes. For these reasons B. succinogenes was selected as the test organism for use in the succeeding experiments conducted to study the metabolic role of valeric acid.

Experiment III was conducted with non-labelled Na-valerate in preparation for studies using labelled valerate in Experiment IV. Trial 1 of Experiment III was designed to obtain data on the growth response of the test organism to the inclusion of valerate and isovalerate, either singly or in combination, in a basal medium containing hydrolysed casein. Response to valerate on deletion of hydrolysed casein from the basal medium was measured in Trial 2. Volatile fatty acid production by B. succinogenes grown in basal media containing glucose as the source of energy was determined in Trial 3.

Materials and Methods

Procedures employed for culture of *B. succinogenes* S85* as a source of inocula and for growth of the organism under the experimental conditions used are described below.

Anaerobiosis

The anaerobic methods of Hungate (1950) were utilized throughout this study for preparing media and growing the culture. All media and the anaerobic solution for washing cell inocula were maintained in sterile rubber-stoppered tubes under an atmosphere of oxygen-free carbon dioxide. Carbon dioxide was freed of oxygen by passage through a column of hot copper filings.

Growth and preparation of washed cell inocula

B. succinogenes S85 for inoculation of experimental media was grown in a glucose-clarified rumen fluid (CRF) medium as described by Bryant *et al.* (1959), except that indigo carmine was replaced by resazurin as the indicator. One hundred ml of the stock culture medium contained the following: 300 mg glucose, 20 ml CRF, 100 mg cysteine-HCl, 400 mg Na_2CO_3, 90 mg $(NH_4)_2SO_4$ and 0.1 mg resazurin.

The CRF was prepared from freshly collected rumen fluid from the fistulated cow used in Experiments I and II. The coarse particles in the rumen fluid as collected were removed by centrifugation, and the resulting supernatant was filtered through a Seitz filter. The clear supernatant was sterilized under CO_2 in an autoclave at 15 lb pressure for 15 minutes and stored at 5°C until used.

The bacteria were transferred daily in the stock culture medium and usually three or more transfers were made before washed cells were prepared. Cells of a 24-hour culture were centrifuged under CO_2 at 3500 r.p.m. for 1 hour in a refrigerated centrifuge. The supernatant was removed with a

*Supplied through the courtesy of Dr. M. P. Bryant, Animal Husbandry Research Division, U.S.D.A., Beltsville, Maryland.

sterile Pasteur pipette, and the pellet was washed once with approximately 5 ml (equal to culture volume) of an anaerobic solution of the nutrients listed in Table 6 minus the casein hydrolysate, glucose and vitamins. After centrifugation the washed cells were diluted to an optical density (OD) of approximately 0.10 in selected test tubes, 13.1 mm in diameter, which had been calibrated at 600 mμ in a Bausch and Lomb Spectronic 20 colorimeter.

Estimation of growth

Five milliliters of experimental medium in each test tube were inoculated with 0.1 ml of the washed cell suspension, and the growth at 37°C was estimated by OD measurement as above. Readings were taken at intervals of 4 - 8 hours, but they were taken more frequently when rate of growth was changing rapidly. Distilled water was used as a blank and no correction was made for the initial reading.

Basal medium

The basal experimental medium (Table 6) was the same as that used by Bryant et al. (1959) except that the sodium salts of n-valeric acid and isovaleric acid were deleted. Solutions of Na_2CO_3, cysteine.HCl and vitamins were sterilized separately by filtration through a Seitz filter and, after equilibration with CO_2, were added to the remaining part of the medium after this had been sterilized in an autoclave and cooled.

Table 6
Composition of basal medium

	mg/100 ml		mg/100 ml
KH_2PO_4	90	Cysteine·HCl	100
NaCl	90	Casein hydrolysate*	200
$(NH_4)_2SO_4$	90	Thiamin·HCl	0.2
$CaCl_2$	9	Pyridoxamine·HCl	0.1
$MgSO_4$	9	Ca-pantothenate	0.2
$FeSO_4 \cdot 7H_2O$	4	Riboflavin	0.2
$MnSO_4 \cdot H_2O$	0.15	Nicotinamide	0.2
$CoCl_2 \cdot 6H_2O$	0.15	p-aminobenzoic acid	0.01
Na_2CO_3	400	Biotin	0.005
Resazurin	0.1	Folic acid	0.005
Glucose	300	Cobalamin	0.0005

*Nutritional Biochemicals Corporation vitamin-free casein hydrolysate (enzymatic)

Trial 1

Object

To study the effect of valerate on the growth of B. succinogenes S85 in the presence and absence of isovalerate.

Experimental

B. succinogenes S85 was incubated in tubes containing 5 ml of the basal medium described in Table 6 with valerate and/or isovalerate treatments in mg/100 ml as shown below.

Na-valerate	Na-isovalerate
3.7	3.3
7.4	3.3
7.4	0.0
0.0	3.3

Two replicates, each with 4 tubes per treatment, were completed. The total incubation period was 96 hours, but only the maximum growth observed in each treatment and the time at which it was recorded are reported.

Results and discussion

The results (Table 7) show that when the valerate level was doubled in the presence of isovalerate, growth of the organism, as measured by optical density of the tubes, increased from 48.2 to 68.2 (41 per cent) in

replicate 1, and from 40.4 to 54.6 (35 per cent) in replicate 2. Somewhat less variation among quadruplicates occurred when the higher level of valerate was used. Very little growth of the organism was obtained when either valerate or isovalerate was excluded from the medium. These results are in agreement with the original demonstration by Bryant and Doetsch (1955) that B. succinogenes S85 has a two-component volatile fatty acid requirement for growth.

Table 7
Effect of Na-valerate and Na-isovalerate on growth of B. succinogenes S85

Added to basal medium (mg/100 ml)		Growth (OD x 100)	
Na-valerate	Na-isovalerate	Replicate 1	Replicate 2
3.7	3.3	48.2 ± 8.6 (21)*	40.4 ± 9.4 (18)
7.4	3.3	68.2 ± 7.1 (21)	54.6 ± 5.9 (18)
7.4	--	11.0 ± 0.8 (57)	9.3 ± 1.4 (42)
--	3.3	8.7 ± 0.8 (57)	9.6 ± 1.7 (50)

*The figures in parentheses refer to the hours of incubation required to reach maximum growth.

Trial 2

Introduction and object

The basal medium used in Trial 1 contained casein hydrolysate. Bryant et al. (1959) have reported that B. succinogenes requires ammonia but not exogenous amino acids for growth. If the radioactivities of amino acids synthesized by B. succinogenes S85 when grown in a medium containing valerate-1-C^{14} were to be used, as planned for Experiment IV, to study the extent or pathway of utilization of valeric acid by this organism it would obviously be advantageous if normal growth of the organism could be obtained in a medium devoid of exogenous amino acids. Trial 2 was therefore conducted to study the effect of valerate on the growth of B. succinogenes S85 in a medium containing no source of preformed amino acids.

Experimental

The basal medium used in Trial 2 was the same as that for Trial 1 (Table 6) except that the casein hydrolysate was deleted and Na-isovalerate was added at a level of 3.3 mg/100 milliliters. The test organism was incubated in tubes containing 5 ml of the basal medium with casein hydrolysate and/or valerate treatments in mg/100 ml as listed below.

Na-valerate	Casein hydrolysate
7.4	200
7.4	--
--	200
--	--

Two tubes for each treatment were incubated in each of the two replicates of the trial.

Results and discussion

The mean results for duplicate tubes in each replicate to 110 hours of incubation are summarized graphically in Figure 5. In the medium containing both valerate and casein hydrolysate, peak growth occurred after 25-40 hours of incubation, whereas maximum growth was not reached until 80-90 hours in the medium containing valerate but no casein hydrolysate. However, the peak growth of the organism in the latter medium was greater than that observed in the medium containing both valerate and hydrolysate. These results show that casein hydrolysate was stimulatory to but not essential for the growth of B. succinogenes S85. Bryant et al. (1959) suggested that the stimulatory effect of amino acids on the initiation of the growth of B. succinogenes S85 may be due to their effect on the Eh of the culture medium or their ability to antagonize inhibitory effects of other compounds or ions in the medium, rather than to their incorporation into cellular protein.

In the tubes containing hydrolysed casein but no added valerate, slight growth in replicate 1 and marked growth in replicate 2 occurred after incubation for approximately 150 hours. el-Shazly (1952b) and Dehority et al.

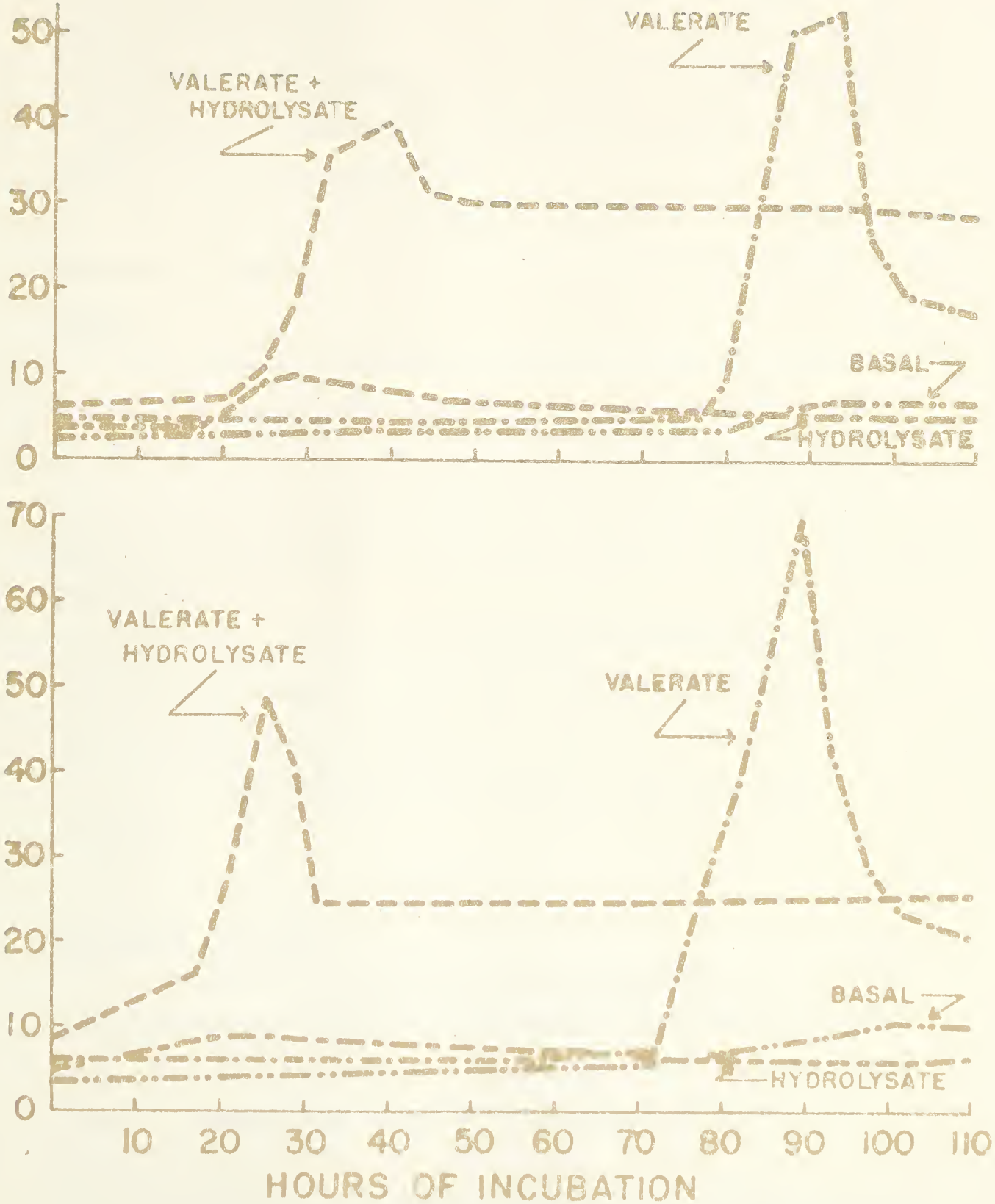

Figure 5. Trial 2 - growth (optical density x 100) of B. succinogenes S85 in basal medium, basal + casein hydrolysate, basal + valerate + casein hydrolysate, and basal + valerate.

(1958) have demonstrated that valeric acid is synthesized by some rumen microorganisms. It is conceivable that in the present experiment enough valeric acid was synthesized from amino acids in the hydrolysate, either through induced metabolic pathways or by a mutant(s) of B. succinogenes S85, to support growth of the organism after the 150-hour lag phase. These possibilities were not investigated.

There was no growth at 200 hours in the absence of both valerate and casein hydrolysate.

Summary

1. In the presence of both valerate and casein hydrolysate, peak growth of B. succinogenes S85 occurred after 25-40 hours of incubation.

2. In the presence of valerate but absence of hydrolysate, no growth occurred during the first 70 hours, but during the next 20 hours growth peaks exceeding those for the tubes containing valerate and hydrolysate were reached.

3. After a lag phase of approximately 150 hours, slight growth in the first and marked growth in the second replicate were observed in the tubes containing casein hydrolysate but no valerate.

4. No growth occurred in the absence of both valerate and hydrolysate.

Trial 3

Introduction and object

Volatile fatty acid production by B. succinogenes grown in rumen juice media has been reported by Hungate (1950), and Bryant and Doetsch (1954). Because the rumen juice contained fatty acids it was impossible to make an unequivocal determination of the fatty acids formed by the organism.

Trial 3 was undertaken to study volatile fatty acid production by B. succinogenes S85 in the presence or absence of casein hydrolysate in a synthetic medium containing glucose as the source of energy.

Experimental Methods

Media

The basal medium used in this trial was the same as that used in Trial 2. Sodium valerate was added at a level of 7.4 mg per 100 milliliters. Two test media were employed. One contained no casein hydrolysate while casein hydrolysate was added to the other at a level of 0.2 g/100 milliliters. Six tubes each containing 5 ml of media were prepared. The medium in tubes 1 and 1C contained no casein hydrolysate and that in tubes 2, 2C, 3 and 3C contained casein hydrolysate.

Inoculation and incubation

The inoculation was carried out with a standard loop. Tube 1 was inoculated with the organism from the original culture in the rumen juice - glucose - cellobiose-agar (RGCA) slant, tube 2 with the organism from tube 1, and tube 3 with the organism from tube 2. Incubations were carried out at 37°C for 96 hours for tube 1, and for 30 hours in the case of tubes 2 and 3. Tubes 1C, 2C and 3C were not inoculated and were stored at 5°C until required for use as controls.

Determination of volatile fatty acids

After fermentation the tubes were centrifuged to precipitate the bacterial cells. One ml of the supernatant, or of the uninoculated medium in the case of the control tubes, was transferred to a 2 ml polyethylene vial and stored at 5°C. The contents of the vials were acidified with 0.05 ml of concentrated HCl before they were analyzed for volatile fatty acids with a gas chromatograph*.

The standard used for qualitative and quantitative determinations of the volatile fatty acids was prepared using pure fatty acids. Five levels of each of these were used for plotting standard curves for the various fatty acids.

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*Ionization Model K-7, Burrell Corporation, Pittsburgh, Pa.

The fatty acids in the experimental and control samples were identified by comparison of their retention times with those of the known fatty acids in the standard (Figure 6). To make quantitative estimates the paper encompassed by the curve for each fatty acid was cut out and weighed. The equivalent weight of fatty acid was read from standard curves prepared from the results obtained with known weights of fatty acids by this procedure. The determinations were carried out in duplicate.

Results and discussion

The results (Table 8) show that acetic acid and propionic acid were the main volatile fatty acids formed under the conditions of this experiment, and they were produced in approximately a 1:3 ratio.

Table 8
Volatile fatty acids formed by B. succinogenes S85 in the presence and absence of casein hydrolysate in a synthetic medium containing glucose as the source of energy

	Tubes 1 and 1C (no hydrolysate)			Tubes 2 and 2C (casein hydrolysate added to medium)			Tubes 3 and 3C		
	1 Fresh Inoculum	1C No Inoc.	Diff.	2 Inoc. from Tube 1	2C No Inoc.	Diff.	3 Inoc. from Tube 2	3C No Inoc.	Diff.
	Volatile Fatty Acids (mmole/100 ml)								
Acetic	.28	-	.28	.29	-	.29	.64	-	.64
Propionic	.97	-	.97	.95	-	.95	1.84	-	1.84
Isovaleric	.003	.001	.002	.001	.001	0	.004	.001	.003
Valeric	.078	.075	.003	.075	.09	—.015	.084	.09	—.006

Hungate (1950) indicated that acetic acid was the chief volatile fatty acid formed by B. succinogenes grown in a rumen liquid-cellulose medium. Bryant and Doetsch (1954) found that in the fermentation of cellulose by B. succinogenes S85 in a rumen fluid medium, acetic and formic were the main volatile fatty acids formed and they believed that the small gain in propionic acid was due to error in analysis.

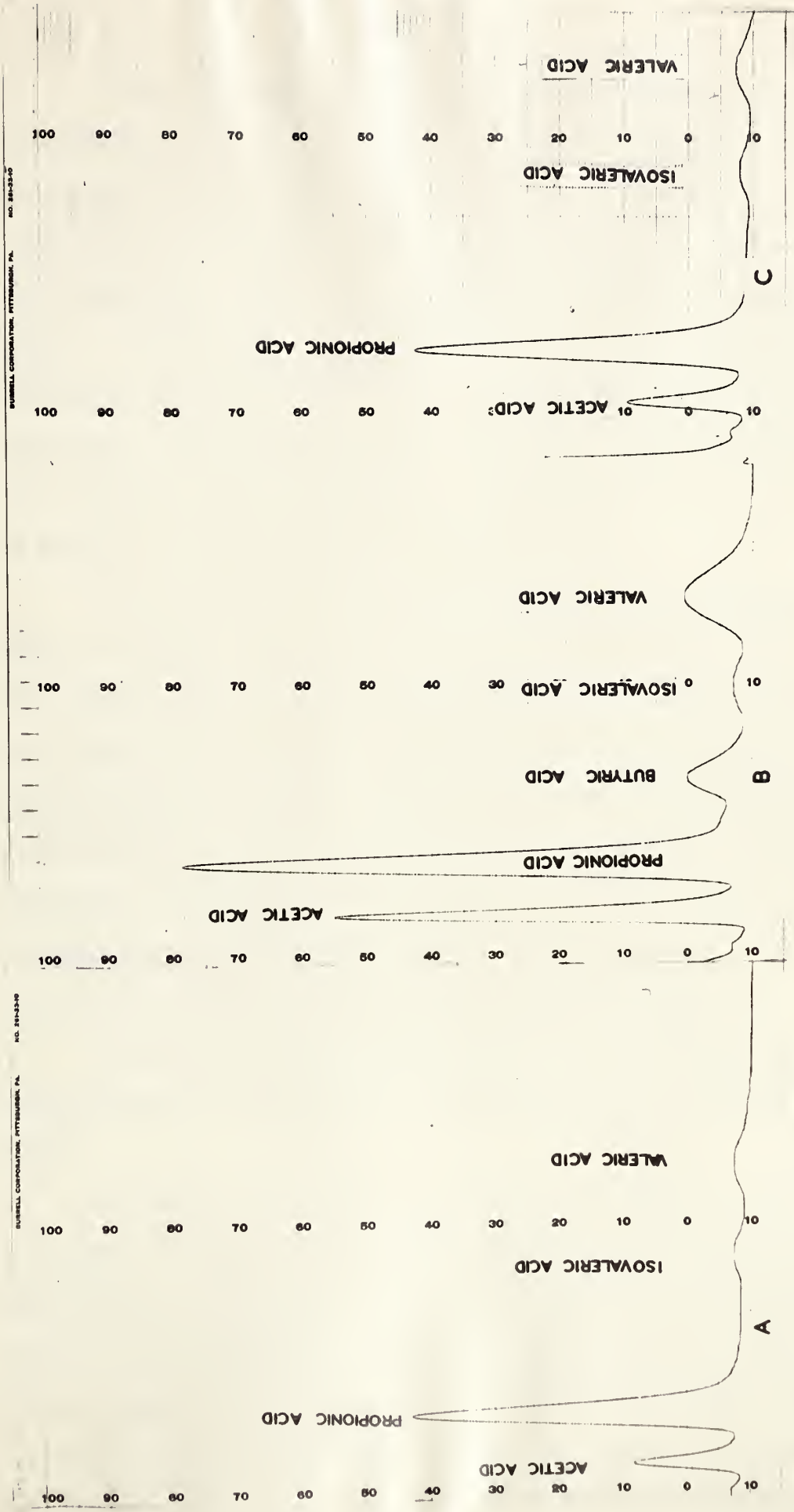

Figure 6. Trial 3 - typical chromatograms of:
A - supernatant of tube 1 (no hydrolysate);
B - standard solution of VFA;
C - supernatant of tube 2 (hydrolysate added).

It has been demonstrated that under in vivo conditions of rumen fermentation the acetate:propionate ratios vary markedly with the nature of the ration. Cellulose apparently favors acetic acid production while starch or glucose favors propionic acid formation in the rumen (Balch and Rowland, 1957; Shaw et al., 1960). B. succinogenes can ferment both cellulose and glucose. Thus the fact that acetic was the acid formed in greatest quantity in the experiments of Hungate and of Bryant and Doetsch, whereas propionic was the main acid produced in the present trial, may be attributable to the fact that they employed a cellulose medium whereas glucose was the only source of energy in the media employed in Trial 3.

In an attempt to confirm this explanation, B. succinogenes S85 was grown in a medium containing 0.3% ball-milled filter paper and 0.1% glucose. Only very limited growth occurred and the amounts of VFA produced were too low to permit their quantitative determination with the gas-liquid chromatographic equipment available. However, to the extent that the chromatograms obtained showed a definite acetic acid peak and little or no change from the base line in the propionic acid position, the results support the thesis that B. succinogenes produces mainly acetic acid when grown in a cellulose medium vs. mainly propionic acid when grown in a glucose medium.

No butyric acid was produced in Trial 3. This observation is in agreement with the results reported by Hungate (1950) and Bryant and Doetsch (1954).

The media used in Trial 3 contained 0.027 mmole of isovaleric and 0.06 mmole of valeric acid per 100 milliliters. By determination (Table 8) the isovaleric acid content of the uninoculated, unincubated control tubes was only 0.001 mmole/100 ml, or less than 4% of the actual content. Corresponding figures for valeric acid were 0.075-0.09 mmole/100 ml by determination or 125-150 per cent of the actual content. It is obvious that the errors of determination for isovaleric and valeric acid at these low concentrations

were such that the results obtained in this trial provide no evidence regarding the degree of utilization of either of these required fatty acids by B. succinogenes S85.

Summary

Propionic acid and acetic acid were the main volatile fatty acids formed by B. succinogenes S85 grown in the synthetic medium containing glucose. No butyric acid was formed by this organism. The data provided no evidence regarding the degree of utilization of either isovaleric or valeric acid for the growth of the organism.

Experiment IV
The Distribution of C^{14} from Labelled Valerate-1-C^{14} in the Cellular Fractions of Bacteroides Succinogenes S85

The results of Experiment II for distribution of C^{14} from labelled valerate-1-C^{14} in the washed cells of rumen microorganisms indicated that valerate-1-C^{14} was extensively degraded to CO_2 and radioactivity incorporated into cellular fractions apparently arose from $C^{14}O_2$ or other radioactive metabolites rather than directly from valerate-1-C^{14}. For reasons presented under Experiment III, employment of inocula from a culture of a single important rumen microorganism, B. succinogenes S85 appeared to offer greater promise for the purpose of this study than use of inocula consisting of washed cells prepared from the rumen contents of a cow. The distribution of C^{14} in the supernatant, lipid, nucleic acid and protein (including individual amino acid) fractions of a culture of B. succinogenes S85 was therefore investigated in the present experiment.

Experimental

Inoculum, medium and fermentation

The anaerobic culture technique and the procedures employed to grow the stock culture and prepare the inoculum were the same as those used in Experiment III. The basal experimental medium, which contained sodium isovalerate but no casein hydrolysate, was prepared and sterilized by the procedures outlined on page 28.

Fermentation was conducted in 125 ml Erlenmeyer flasks. Ninety-eight ml of basal medium and 9.7 mg and 4 mg of Na-valerate-1-C^{14} were added to the flasks for replicates 1 and 2 respectively. The flask was then sterilized in an autoclave at 15 lb pressure for 15 minutes. After cooling, the original rubber stopper was replaced with a two-hole rubber stopper fitted with glass inlet and outlet tubes to which rubber tubes were attached.

The inlet tube was connected to a source of oxygen-free CO_2 and the medium was flushed with CO_2 for 30 minutes. Two ml of a suspension of washed cells of B. succinogenes S85 were added and the flushing with CO_2 repeated for 2 minutes after which the inlet and outlet tubes were closed with strong clamps. Incubations were carried out in a water bath at 37°C for 96 and 144 hours for replicates 1 and 2 respectively.

At the end of the incubation period a 30 ml test tube containing 20 ml of saturated $Ba(OH)_2$ solution was connected to the outlet tube of the Erlenmeyer, and the clamp on the tube was released slowly. Approximately 100 ml of air were introduced with a syringe into the fermentation flask through the inlet tube to replace the gas in the flask. The CO_2 formed during fermentation was collected in the $Ba(OH)_2$ trap.

Fractionation

The fractionation procedures employed were similar to those used in Experiment II. The cells and supernatant were separated by centrifugation. Volatile and non-volatile constituents of the supernatant were separated by steam distillation under acid conditions of 5 g and 11.6 g portions of the supernatant of replicates 1 and 2 respectively.

The cells were chemically fractionated into lipid, nucleic acid and protein portions by the procedures described on page 16 to yield supernatant and wash, lipid extract, nucleic acid extract and protein hydrolysate. The lipid portion was further treated by distillation under acid conditions on a water bath. The residue of the lipid portion was dissolved in a mixture of 2 ml water and 5 ml n-hexane (Katz and Chaikoff, 1954). The extraction was repeated 3 times until the residue was completely dissolved. After shaking the water-hexane emulsion was separated by centrifugation. The upper layer, denoted as the n-hexane soluble fraction, was collected with a Pasteur pipet.

Estimation of radioactivity

The methods used for determination of the activity of the various fractions were similar to those used in Experiment II, except that smaller samples of known weight rather than volume were spread on the planchets. A 'Micromil' window gas-flow counter* instead of the windowless counter was used.

Chromatographic separation and determination of amino acids

Both one- and two-dimensional paper chromatographic techniques were employed for separating the amino acids present in the hydrolysate. One-dimensional chromatograms were prepared as in Experiment II except that the descending instead of ascending chromatographic technique was used. Chromatograms were prepared by the method of Levy and Chung (1953), which involves the preparation of both one- and two-dimensional chromatograms on the same sheet. Whatman No. 1 sheets, measuring 46 x 57 cm, were used and chromatograms of the hydrolysate and a control solution of amino acids were run on the same sheet. One-dimensional descending chromatograms were prepared in a chromatograph cabinet at room temperature over periods of 18 to 20 hours. After drying in the air the one-dimensional chromatograms were sprayed with 1% ninhydrin in n-butyl alcohol to detect the amino acids.

The unstained strips for two-dimensional chromatograms were cut off. The area of the amino acid spots was covered by placing each sheet between two 1.5 x 25-inch strips of plate glass held together tightly with elastic bands. The exposed paper was sprayed with borate buffer (pH 9.3), removed from the glass and dried at 40°C for one hour.

The sheets were then made cylindrical by folding lengthwise and stapling at the junction of the ends. The second dimension (ascending) chromatograms were run by placing the cylinders on edge in a glass tank

*Nuclear Chicago Model D47

containing, as the mobile phase, a layer of phenol, borate buffer (pH 8.3) and meta-cresol. The aqueous phase was supplied by placing a beaker containing 250 ml of borate buffer (pH 9.3) and 8 ml of the mobile phase in the tank adjacent to the chromatograph sheet.

When the run was completed (7-9 hours) the chromatogram was dried at 40°C for 2 hours and sprayed with a mixture of 50 ml of 0.1% ninhydrin in ethyl alcohol, 2 ml of collidine, and 15 ml of glacial acetic acid. While still wet it was held 2 to 3 inches above a hot plate for 1 to 3 minutes until color development was complete.

The amino acids were identified by comparing their Rf values with those of amino acids in the reference solution chromatographed on the same sheet. The identification was confirmed by the positions and the colors of the amino acid spots from the hydrolysate on the two-dimensional chromatograms.

Radioautographs

Radioautographs were made from paper chromatograms employing Ilford 'no screen' X-ray film and an exposure time of 4 weeks as in Experiment II. The relative activities of the radioactive amino acid spots shown on the radioautographs were determined by cutting out the spots with scissors, placing each on a planchet and measuring the radioactivity in the 'Micromil' window gas-flow counter.

Results

The following yields, in grams, were obtained on fractionation of the cultures of replicates 1 and 2 respectively: wash, 75.5 and 60.4; lipid extract, 26 and 25.5; nucleic acid extract, 15 and 20; protein hydrolysate, 2.6 and 1.8. The distribution of radioactivity in the major fractions of cultures of *B. succinogenes* S85 grown in the synthetic medium containing valerate-1-C^{14} is summarized in Table 9. The total activity recovered in the various fractions was greater than that of the whole culture.

This apparent increase in radioactivity undoubtedly arose from the accumulated errors of sampling and counting.

Table 9
Distribution of C^{14} from valerate-1-C^{14}
in cultures of B. succinogenes S85

Fractions	Replicate 1		Replicate 2	
	Radio-activity (cpm)	% of whole culture	Radio-activity (cpm)	% of whole culture
Whole culture	20,297,000	100	6,715,000	100
Supernatant and wash	20,571,000	101.3	5,907,000	88.0
CO_2	2,000	--	70,000	1.0
Washed bacteria cells	2,110,000	10.3	2,591,000	38.5
Lipid	1,545,000	7.6	1,512,000	22.5
Nucleic acid	96,000	0.4	69,000	1.0
Protein	469,000	2.3	1,010,000	15.0
Total activity of fractions	22,683,000	111.6	8,568,000	127.5

More than 100% of the activity in the culture of replicate 1 and approximately 90% of that in replicate 2 was recovered in the supernatant. In relation to that in the whole culture, the activity found in the $Ba(OH)_2$ trap amounted to only 0.01% and 1% in replicates 1 and 2 respectively. The cellular incorporated activity (10-30%?) appeared mainly in the lipid and protein fractions, and the activity of the nucleic acid fraction was relatively low in both replicates.

When the supernatant of replicate 1 was steam distilled under acid conditions, more than 100% of the activity originally found in the supernatant was recovered in the volatile fraction, and the activity remaining in the residue was about 9% of the total activity (Table 10); for replicate 2, the corresponding values were approximately 43 and 8 per cent. The results for the replicates were thus in agreement to the extent that a major portion of the activity of the supernatant was in the steam volatile fraction. In Experiment II, 85% of the radioactivity of the supernatant of cultures of

washed rumen cells was lost, presumably as $C^{14}O_2$, when acid was added whereas, for cultures of B. succinogenes S85, while about 50% of the activity in the supernatant was apparently lost on acidification of replicate 2, there was no loss in replicate 1. In general the results suggest that the major portion of the radioactivity in the supernatant may have been present as intact valerate.

Table 10
Distribution of C^{14} in the supernatant

Fractions	Replicate 1		Replicate 2	
	Radio-activity (cpm)	% of super-natant	Radio-activity (cpm)	% of super-natant
Supernatant*	1,142,000	100	655,000	100
Distillate	1,207,000	105.7	283,000	43.2
Residue	99,000	8.6	51,000	7.8
Total activity of fractions	1,306,000	114.3	334,000	51.0

*5 g of supernatant of replicate 1 and 11.6 g of supernatant of replicate 2 were used for fractionation.

An attempt was made to separate the fatty acids in the volatile fraction of the supernatant of replicate 1 by means of a silicic acid column (Keeney, 1955) using a known volatile fatty acid mixture added to the sample as a carrier. The reproducibility of results was poor but the major portion of the recovered activity of the sample was found in the C_5 (or higher?) fatty acid fraction. There was very little activity in the propionic or acetic acid fractions.

The major portion of the activity of the lipid fraction was recovered in the non-volatile, n-hexane soluble fraction (Table 11).

The main radioactive compound(s) in the lipid fraction of both replicates was non-volatile and insoluble in water but soluble in n-hexane. This suggests that the radioactive carbon atoms were incorporated into true lipid(s) rather than compounds such as free amino acids, Krebs cycle intermediates or phosphorylated carbohydrates, which could also be present in ether-ethanol extracts of the lipid fraction (Abelson et al., 1952).

Table 11
Distribution of C^{14} in the lipid fraction

Fractions	Replicate 1 Radio-activity (cpm)	Replicate 1 % of lipid	Replicate 2 Radio-activity (cpm)	Replicate 2 % of lipid
Lipid*	1,545,000	100	773,000	100
Distillate	4,000	0.3	27,000	3.5
Residue	1,276,000	82.6	311,000	40.2
n-hexane extract	1,180,000	76.4	297,000	38.4
Water extract	96,000	6.2	14,000	1.8
Total activity of fractions	1,280,000	82.9	338,000	43.7

*26 g and 13 g of the lipid fraction of replicates 1 and 2 respectively were fractionated.

When the protein hydrolysates of replicates 1 and 2 were chromatographed on the same sheet, the radioactive ninhydrin-reactive compounds of both replicates had the same Rf values. The radioautographs of the one-dimensional chromatograms of the protein hydrolysate of replicate 1 indicate that the radioactive carbon atoms were incorporated mainly into six ninhydrin-reactive compounds (Figure 7). Similar results were revealed by the radioautograph of the two-dimensional chromatogram (Figure 8). The labelled compounds were identified as lysine, arginine, aspartic acid, glutamic acid, proline, and leucine (Table 12).

Table 12
Rf values of reference and radioactive amino acids run on the same one-dimensional chromatograms in butanol-acetic acid-water

Amino acids	Determination 1 Refer-ence	Determination 1 Radioactive amino acid	Determination 2 Refer-ence	Determination 2 Radioactive amino acid	Determination 3 Refer-ence	Determination 3 Radioactive amino acid
Lysine	.16	.15	.15	.17	.17	.16
Arginine	.18	.18	.20	.20	.20	.19
Aspartic acid	.23	.22	.25	.24	.23	.22
Glutamic acid	.30	.31	.31	.33	.32	.30
Proline*	.45	.43	.45	.44	.45	.43
Leucine	.71	.71	.72	.72	.70	.70

*Proline was not included in the reference solution. The typical yellow-colored spot shown on the two-dimensional chromatogram of determination 3 was used to identify proline.

Abbreviations - Figures 7 and 8

ALA - alanine
ARG - arginine
ASP - aspartic acid
CYS - cystine
GLY - glycine
GLU - glutamic acid
HIS - histidine
ILEU - isoleucine
LEU - leucine
LYS - lysine
PHE - phenylalanine
PRO - proline
SER - serine
THR - threonine
TYR - tyrosine
VAL - valine

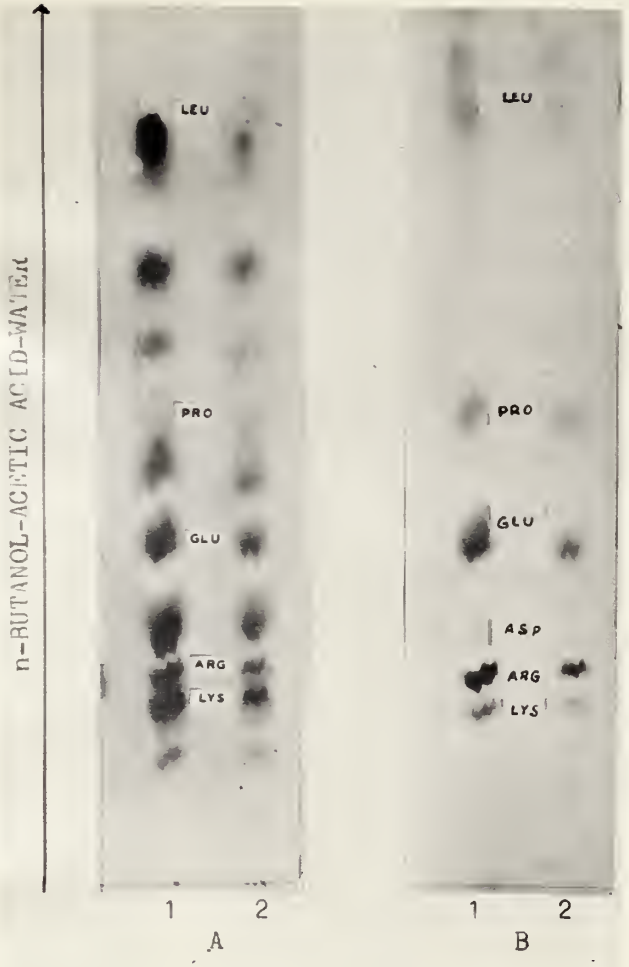

Figure 7. (A) One-dimensional chromatogram; 75 µl of the protein hydrolysate of replicate 1 were used for 1 and 25 µl for 2.
(B) Radioautograph of (A).

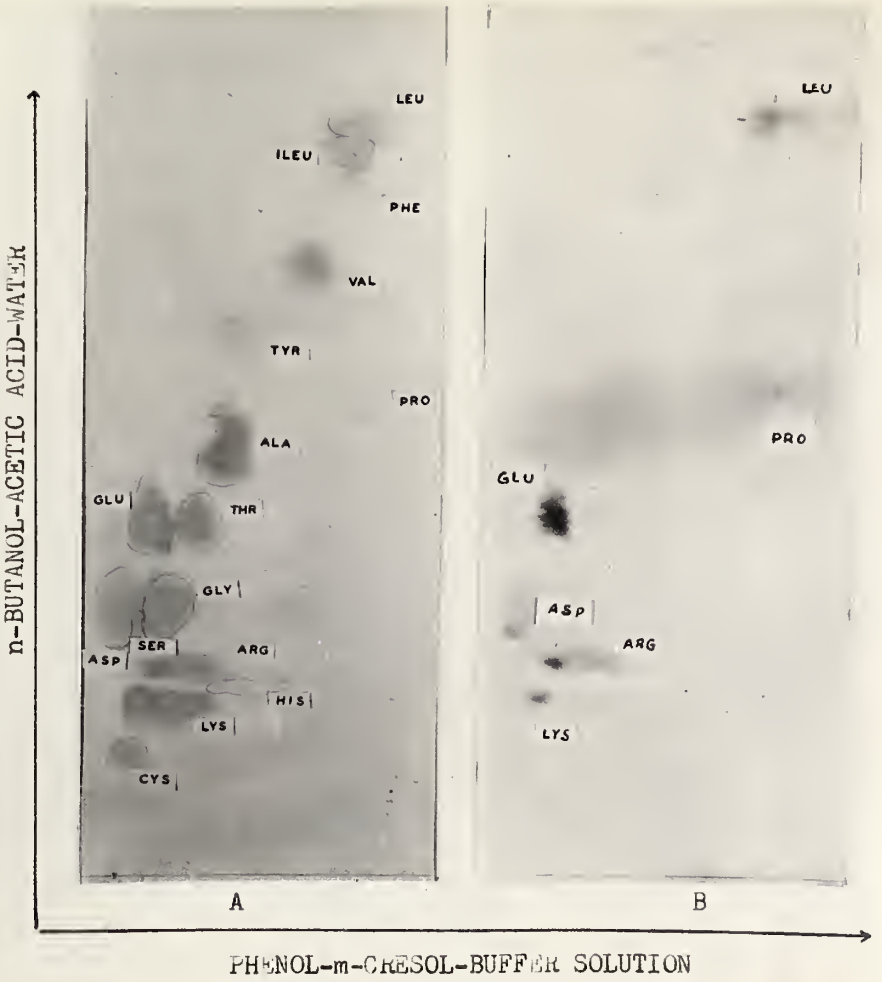

Figure 8. (A) Two-dimensional chromatogram corresponding to Figure 7 (A).
(B) Radioautograph of (A).

The results obtained from counting the active spots from the one-dimensional chromatograms of replicates 1 and (Table 13) were in agreement with those shown on the radioautographs (Figures 7 and 8). Arginine, glutamic acid and proline were the most heavily labelled amino acids. In terms of specific activity relative to arginine, the remaining active amino acids ranked proline-glutamic and lysine-leucine-aspartic.

Table 13
Relative radioactivities of amino acids

	Radioactivity (cpm)		Activity relative to arginine		Moles relative to arginine*	Specific activity relative to arginine	
Replicate No.	1	2	1	2		1	2
Lysine	113	244	33	28	1.99	16	14
Arginine	346	859	100	100	1.00	100	100
Aspartic acid	53	384	15	44	3.08	5	14
Glutamic acid	260	1544	75	179	2.83	26	63
Proline	163	537	47	62	0.89	53	70
Leucine	83	277	23	32	2.15	11	14

*Data from Table 14

The amino acid composition of the protein hydrolysate of B. succinogenes S85 was determined by ion exchange chromatography*. The hydrolysate used for this determination was prepared from a culture of the organism grown in the same medium and under the same conditions as those used in the tracer studies of the present experiment, but non-labelled valerate was used instead of valerate-1-C^{14}. The method used for preparing the hydrolysate was similar to that used in preparing the hydrolysate for the paper chromatograms. The nitrogen content of the hydrolysate was determined in duplicate by the micro-Kjeldahl method (A.O.A.C., 1960).

As shown in Table 14, the amino acid composition of the protein hydrolysate of B. succinogenes S85 grown in the synthetic medium was similar to that of mixed ruminal bacteria as reported by Weller (1957). Essentially the same amino acid pattern was found (not reported in this thesis) in a

*Beckman Spinco Amino Acid Analyzer, Model 120.

protein hydrolysate of B. succinogenes S85 grown in a glucose-clarified rumen fluid medium.

Table 14
Amino acid composition of the hydrolysates of B. succinogenes S85 and of hydrolysates of mixed rumen microorganisms

	B. succinogenes S85			Mixed rumen microorganisms*
Amino acid	Micro-moles	Moles relative to arginine	Amino acid nitrogen of total nitrogen %	Amino acid nitrogen of total nitrogen %
Lysine	0.438	1.99	8.1	7.5 - 8.2
Histidine	0.096	0.44	2.6	2.6 - 3.0
Ammonia	1.264	5.74	11.7	--
Arginine	0.220	1.00	8.1	8.6 - 9.3
Aspartic acid	0.678	3.08	6.3	6.7 - 6.8
Threonine	0.292	1.33	2.7	3.5 - 3.8
Serine	0.276	1.25	2.6	2.5 - 3.0
Glutamic acid	0.622	2.83	5.8	6.6 - 7.5
Proline	0.196	0.89	1.8	2.1 - 2.8
Glycine	0.510	2.39	4.7	5.9 - 6.3
Alanine	0.652	2.96	6.0	6.4 - 6.5
Half cystine	nil	nil	nil	0.7 - 0.8
Valine	0.441	2.00	4.1	4.4 - 4.5
Methionine	0.075	0.34	0.7	1.5
Isoleucine	0.341	1.55	3.2	3.6 - 3.8
Leucine	0.473	2.15	4.4	4.5 - 4.7
Tyrosine	0.131	0.59	1.2	2.0 - 2.2
Phenylalanine	0.241	1.09	2.3	2.3 - 2.5

*Weller (1957)

Discussion

Of the known deamination reactions only aspartic deaminase or aspartase and glutamic deaminase or glutamic dehydrogenase are reversible. Thus it is generally considered that it is primarily through the reactions catalyzed by these enzymes that ammonia may be converted into amino groups (Wilson and Burris, 1947; Oginsky and Umbreit, 1959). The carbon skeletons for the synthesis of amino acids in such a system would come from fumarate and α-ketoglutarate. However, in a study of the incorporation of randomly labelled C^{14}-glucose in E. coli, Roberts et al. (1955) demonstrated that α-ketoglutarate is not a precursor of glutamic acid. Hence the incorporation of C^{14} from glucose into amino acids if this generally accepted pathway

were followed would be highest in aspartic with lesser amounts in glutamate and arginine. In addition, C^{14} derived from $NaHC^{14}O_3$ or $C^{14}O_2$ was incorporated during protein synthesis mainly into aspartate, glutamate and arginine. The level of incorporation was higher in aspartic than in glutamic acid (Huhtanen et al., 1954; Roberts et al., 1955).

However, in the present experiment a different metabolic pathway appeared to be involved. There was a relatively low recovery of C^{14} in aspartate compared to glutamate, arginine, and proline. Since the specific activity of aspartate was comparatively low, it seems logical to suggest that the aspartase reaction is not a major pathway in the utilization of valeric acid for protein synthesis by B. succinogenes S85 under these conditions.

The major pathway for protein synthesis in this experiment seems to be closely connected with the metabolism of arginine, proline and glutamic acid. Enzymes involved in the interconversion of these amino acids are known in the mammalian urea cycle. These reactions are illustrated in Figure 9. Arginine may be converted to ornithine which, when the terminal amino group is deaminated, forms intermediates which can lead to glutamic acid and proline. By another series of reactions, proline may be converted to ornithine which, in turn, may give rise to arginine. If these or similar enzyme systems exist in B. succinogenes S85, the interconversion of the three amino acids with the highest specific activities can be explained. There still remains the problem of how n-valeric is incorporated into these amino acids.

Dehority et al. (1958) reported that the metabolic fate of proline in rumen microorganisms is different from that in other bacteria. It apparently undergoes reductive ring cleavage and deamination at the δ-position to form valeric acid (Figure 9). If this is a reversible process, B. succinogenes S85 may utilize valeric acid to synthesize proline.

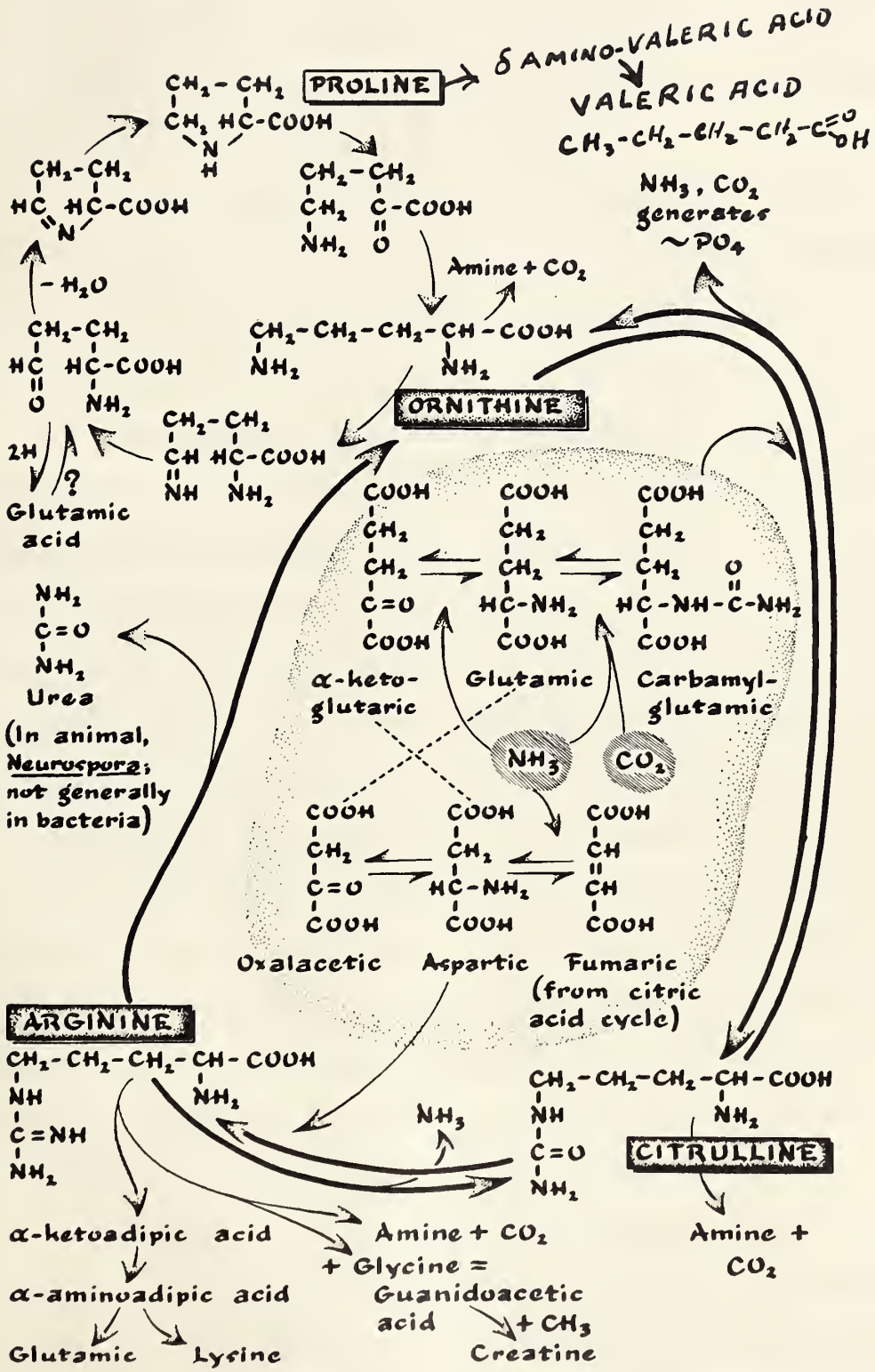

Figure 9. The "urea cycle" and related reactions. (Oginsky and Umbreit, 1959)

The reactions illustrated in Figure 9 do not fully explain all of the observations in the present experiment, especially the relatively high specific activity of arginine but it seems logical to assume that the incorporation of n-valerate into amino acids is effected through reactions which are closely related to this scheme.

In Neurospora, lysine may be synthesized by the amination of α-amino adipic acid which may be derived from arginine via α-keto adipic acid. If a similar pathway exists in B. succinogenes S85, it would explain the labelling of lysine with a lower specific activity than that of arginine.

The incorporation of C^{14} from n-valerate into leucine is difficult to explain. Leucine has been shown to arise from iso-valerate in Ruminococcus flavefaciens (Allison et al., 1959) and from pyruvate via α-keto isovalerate in E. coli (Roberts et al., 1955). Neither of these pathways is adequate to explain the results of the present experiment. Leucine synthesis is more easily explained if some fragmentation of n-valerate is assumed than if the intact carbon skeleton is considered to be the precursor of this amino acid. The incorporation of C^{14} into the lipid fraction indicates that some metabolic process not considered in the discussion of amino acid synthesis was involved. Such a process would probably not involve the production of CO_2 because of the low activity in this fraction, but could involve fragmentation of the n-valerate which would supply intermediates for leucine synthesis. Such a mechanism could also account for the incorporation of C^{14} into aspartic acid.

In general, the above hypotheses concerning the utilization of valerate-1-C^{14} for amino acid synthesis are based on the assumption that the intact carbon skeleton of n-valeric acid, rather than 2- or 3-carbon derivatives, is used for amino acid synthesis by B. succinogenes S85. Bryant and Doetsch (1955) demonstrated that there was no growth response of B. succinogenes to acetic or propionic acid. This observation, together

with the finding that there is a growth response to n-valeric and iso-valeric acid even in the presence of glucose and casein hydrolysate, seems to support the basic assumption. However, as has been indicated above, some pathways may require 2- or 3-carbon units.

A more detailed study of the intermediate metabolites is necessary before any conclusive suggestions can be made concerning the method of incorporation of n-valeric acid in B. succinogenes S85.

Summary

1. Radioactive carbon from valerate-1-C^{14} was incorporated into cellular fractions of B. succinogenes S85 - mainly in the lipid and protein portions.

2. The activity found in the protein portion mainly appeared in lysine, arginine, aspartic acid, glutamic acid, proline and leucine; arginine, proline and glutamic acid had the highest relative specific activities among these amino acids. The utilization of valerate-1-C^{14} by B. succinogenes S85 in the synthesis of these amino acids may have occurred via proline through the reverse pathway to that followed in other rumen microorganisms studied to date. It is also possible that an unknown metabolic pathway between valeric acid and arginine may exist in B. succinogenes S85.

GENERAL DISCUSSION

Valeric acid significantly increased both the per cent of cellulose digested and the weight of TCA-N formed in the artificial rumen fermentation. The results of the observed stimulatory effect of valeric acid is in agreement with those reported by Bentley *et al.* (1955) and Cline *et al.* (1958). They found that the increase of cellulose digested was coupled with the increase of TCA-N formed by rumen microorganisms in the presence of valeric acid in the medium.

The stimulatory effect of valeric acid was mainly on the growth of ruminal cellulolytic bacteria. In the presence of valeric acid in the medium the increase of per cent cellulose digested was to a greater extent than the increase of weight of TCA-N formed. This indicates that the increase in microbial population was mainly to increase number of cellulolytic bacteria such as *B. succinogenes* which requires valeric acid for growth. This finding would agree with the postulation of Cline *et al.* (1958), that either ruminal amylolytic bacteria do not require valeric acid or their requirement is much lower than that of cellulolytic bacteria.

It was noted that the labelled valerate-1-C^{14} was extensively degraded to $C^{14}O_2$ by the washed cells of rumen microorganisms. When valerate-1-C^{14} was incubated with the washed cells more than 40 per cent of the total recovered activity was found in the $Ba(OH)_2$ trap, the activity recovered in the various cellular fractions was only 4% of the activity in the whole culture and the major portion of the activity in the supernatant was apparently in the form of radioactive carbonate ($C^{14}O_3$). A second trial showed that the activity noted in the cellular fractions arose from the radioactive metabolite(s), mainly $C^{14}O_2$, rather than from the intact valerate-1-C^{14}.

In the rumen valeric acid is synthesized by certain rumen microorganisms. During the incubation with the washed cells, the intact valerate-1-C^{14} pool may have been diluted with synthesized non-labelled valeric acid as well as being degraded to $C^{14}O_2$. Under this situation if certain ruminal microorganisms would require valeric acid for synthesis of the cellular constituents there was little probability that the C^{14} from the valerate-1-C^{14} would be incorporated into the cellular constituents in a significant amount. This complex biological system that existed in the mixed flora would be avoided by employing a pure culture of appropriate ruminal bacteria for the tracer study. B. succinogenes S85 was selected for this purpose.

Valeric acid was an important growth factor for B. succinogenes S85. The observed growth response of this organism to valerate was in agreement with that reported by Bryant and Doetsch (1955) and Bryant et al. (1959). The peak growth of the organism was not increased by the presence of casein hydrolysate in the medium. This would agree with the results of Bryant et al. (1959) and Bryant and Robinson (1961b), who found that B. succinogenes does not require amino acids for growth.

Cline et al. (1958) indicated that cellulolytic bacteria seemed to utilize valeric acid, and amylolytic bacteria seemed to synthesize valeric acid. The results found in a trial to study the volatile fatty acid production by B. succinogenes S85 were not satisfactory to indicate the degree of utilization of valeric acid by the organism.

There was little radioactive CO_2 formed from valerate-1-C^{14} by B. succinogenes S85. In contrast to the experiment with the washed cells of rumen microorganisms only 1% or less of the activity appeared in the $Ba(OH)_2$ trap. This would agree with the observation of Hungate (1950) that there was no excessive CO_2 formation during the fermentation of B. succinogenes.

The major portion of the activity in the supernatant was recovered in the volatile fraction in which the more activity was recovered in the high fatty acid fraction rather than 2- or 3-carbon fatty acid fractions by silicic acid column chromatography. This suggests that the activity remaining in the supernatant was mainly in the form of intact valerate-1-C^{14}.

The percentage of the activity incorporated into the cellular fractions of B. succinogenes S85 was as much as nine times that of the activity incorporated into the cellular fractions of the washed cells of rumen microorganisms. The incorporated activity was mainly recovered in the lipid and protein portions.

The activity in the protein portion mainly appeared in lysine, arginine, aspartic acid, glutamic acid, proline and leucine. Arginine, proline and glutamic acid had the highest relative specific activities among these active amino acids. The low recovery of the activity in the $Ba(OH)_2$ trap, and the unequal labelling of glutamic and aspartic acid indicate that the entry of C^{14} from valerate-1-C^{14} into these amino acids would not be by way of the aspartase reaction. Because arginine, proline and glutamic acid had the highest relative specific activities it seems logical to assume that the incorporation of valerate-1-C^{14} into these amino acids is effected through reactions which are closely related to the urea cycle. However, it is far from clear how valeric acid might be incorporated into the active amino acids. It is possible that the entry of valerate-1-C^{14} into the amino acids via δ-amino valeric acid and proline followed the reverse pathway of proline degradation in the rumen, or an unknown metabolic pathway between valeric acid and arginine might exist in B. succinogenes S85. It is apparent that a more detailed study of the intermediate metabolism is necessary before any conclusive suggestion can be made regarding the mode of entry of C^{14} from valerate-1-C^{14} into

these amino acids and the major metabolic role of valeric acid in B. succinogenes S85.

CONCLUSIONS

1. Valeric acid significantly increased both the per cent of cellulose digested and the weight of TCA-N formed in the artificial rumen fermentation by the washed cells of rumen microorganisms. The stimulatory effect was mainly on the growth of ruminal cellulolytic bacteria.

2. The valerate-1-C^{14} was extensively degraded to radioactive $C^{14}O_2$ by the washed cells. It was impossible to study the metabolic role of valeric acid, using valerate-1-C^{14} as a tracer, under this complex biological system in the mixed population of rumen microorganisms.

3. Valeric acid was a growth factor for B. succinogenes S85. The results were not satisfactory to indicate the degree of utilization of valeric acid by B. succinogenes S85.

4. Approximately 10 to 30% of the activity in the whole culture was incorporated into the cellular fractions of B. succinogenes S85. The incorporated activity mainly appeared in the lipid and protein portions. In the protein portion the activity was principally recovered in lysine, arginine, aspartic acid, glutamic acid, proline and leucine. Arginine, proline and glutamic acid had the highest relative specific activities among the active amino acids. The utilization of valerate-1-C^{14} by B. succinogenes S85 for synthesis of these amino acids appeared to be closely related with reactions involved in the urea cycle.

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